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Chemical and Biological Triggers for the Improved Intercalation and Release of Glucose Oxidase (GO) in the Galleries of α -Zirconium Phosphate Nanolayers for a Noninvasive Oral Alternative to Insulin Drug Therapies

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Chemical and Biological Triggers for the Improved Intercalation and Release of Glucose
Oxidase (GO) in the Galleries of α -Zirconium Phosphate Nanolayers for a Noninvasive Oral
Alternative to Insulin Drug Therapies

(Via two systems: α -Zrp-Glucose Oxidase-Divalent Metal Ion (Mg^{2+} , Ca^{2+} , and Ba^{2+}) and α -Zrp-
Glucose Oxidase-Cationized Bovine Serum Albumin (BSA))

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Biological Sciences University Honors Scholar Thesis
May 2013

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1. Abstract

Noninvasive treatment alternatives for diseases from diabetes to hypercholesterolemia are at the cutting edge of research. Although many noninvasive drug delivery routes, including oral nasal, dermal, buccal, and pulmonary have been investigated no one route has been fully approved for patient use by the *Food and Drug Administration (FDA)* standards as an alternative to current needle dependent subcutaneous insulin delivery.¹⁰ See table 1 in *Recent Challenges in Insulin Delivery Systems: A Review* for a summation of the current noninvasive insulin drug delivery systems along with their stage of FDA approval.¹² This thesis focuses on two novel oral protein drug delivery systems α -Zr(IV) phosphate (α -ZrP)-protein-Divalent Metal Ion and α -Zr(IV) phosphate (α -ZrP)-protein- cationized bovine serum albumin BSA. Glucose oxidase (GO) serves as a model protein in this thesis for noninvasive alternatives to lowering blood sugar in type one and two diabetes patients. However, instead of encapsulating GO, an oral drug treatment for diabetes, alternative proteins can also be encapsulated as oral drug treatments for ailments such as hypercholesterolemia This experiment systematically tests what ionized version of BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5), or divalent metal ion (Mg (II), Ca (II), or Ba (II)) intercalated into ZrP results in maximal GO binding and GO release in the intestinal region of the GI tract.

Binding studies showed 10 μ M of all forms of cationized BSA tested as well as 50 μ M [Mg^{2+}], between 25 μ M and 50 μ M [Ca^{2+}], and 50 μ M [Ba^{2+}] maximized binding of GO protein in ZrP. (under conditions are as stated in the experimental section) Least to most binding maximization was seen as cationization was increased from BSA-TEPA (0) up to BSA-TEPA (+5). These aforementioned divalent metal ion or cationized BSA forms are thus best suited for capturing proteins to deliver in noninvasive protein drug delivery systems. Zeta Potential studies, as supported by agarose gel electrophoresis, confirmed successful cationization of BSA samples utilized in the experiment. Additionally, zeta potential studies showed Ca^{2+} , Ba^{2+} , and Mg^{2+} increased zeta potential or ZrP's surface charge. (ordered in least to most capacity to increase zeta potential) Similar to the cationized versions of BSA, it is thus expected that alpha-ZrP present with divalent metal ions as compared to with no divalent metal ions will act as a stronger glue that allows maximal GO binding. (see discussion for theory) Release studies showed retention increased upon GO addition to ZrP in presence of Mg (II), Ba (II), native BSA (-25), BSA-TEPA (-13), BSA-TEPA(0), BSA-TEPA-(+5). (figures 12 and 14) This suggests a drug delivery system composed of drug- alpha-ZrP-Mg (II), or drug- alpha- native BSA (-25, drug-alpha-ZrP-BSA-TEPA (-13), drug- alpha-ZrP-BSA-TEPA (0), or drug- alpha-ZrP-BSA-TEPA (+5) is best to maintain a drug's activity while being transported through a similar environment (pre-Intestinal Simulation [NaCl] 0g/L, pH 7.0, 25 °C) before entering a desired site of drug release. (for example the intestines in the case of GO for diabetes treatment) Moreover, release studies showed retention increased upon GO addition to ZrP in presence of Ca (II), Ba (II), native BSA (-25) and BSA-TEPA (+5). (figures 16 and 18) This suggests a drug delivery system composed of drug- alpha-ZrP-Ca (II), or drug- alpha-Ba (II), drug- alpha-ZrP-native BSA (-25), or drug- alpha-ZrP-BSA-TEPA (+5) is best to maintain a drug's activity if that drug must be released in the intestines.

2. Acknowledgements

- To Dr. Challa Kumar for pushing me to make connections, advising me along my long journey, and welcoming me into the lab...He has solidified in me the desire to constantly question and search for something more.
- To the Day of Pride Scholarship donors and selection committee for allowing me to pursue a higher education. Thanks to the generosity of the donors... I am not only the first generation in my family to attend college, but to do so on a full scholarship. To that I am forever grateful.
- To the Kumar Lab for always being there to answer my questions and advise me during each and every experiment.
- To my high school teachers from Portland, CT for believing in me.
- To the Honors Program for giving me the opportunities a first generation college student like myself could only dream of having access to.
- Most importantly... to my father Anwar Afrede, mother Shahana Afrede, and older brother Momin Afrede that have served as my motivation. Thank you for instilling the importance of humility, work effort, gratitude, and generosity into my being.

3. Introduction

3.1 Goal:

The main goal of the project is to stabilize glucose oxidase (GO) against digestion and extreme pH conditions by its intercalation in the galleries of Zr(IV) Phosphate layered inorganic nanomaterial, for applications in non-invasive therapies. See table 1 for paper summation.

Ultra-stable preparations of GO that can survive the gastro-intestinal conditions can be useful to convert intestinal glucose to gluconic acid and lower glucose levels in the gut. Since, the intestinal walls are permeable to glucose, this glucose gradient across its walls would allow more glucose to enter the intestines from the bloodstream, lowering the blood glucose by noninvasive modality. The goal is to make noninvasive GO by its intercalation into the galleries of α -Zr(IV)phosphate (α -ZrP), and protect the enzymes in the stomach and intestines. The galleries of α -ZrP are narrow and they are not expected to permit hydrolysis of the intercalated proteins by photolytic enzymes such as trypsin or pepsin. The layers serve as a physical barrier and prevent access to the intercalated proteins and enzymes. The extreme pH of the stomach (pH<1) is another concern, as acid can catalyze the hydrolysis of amine bonds present in proteins and enzymes. The local pH of intercalated GO will be stabilized by intercalating cationized BSA or metal cations along with GO such that the cationized BSA or metal salts would serve as buffers and prevent drastic pH changes. Thus, we hope that this research would help usher in alternative methods to lower blood glucose levels in a safe and effective manner.

Goal:

- stabilize glucose oxidase (GO) against digestion and extreme pH conditions by its intercalation in the galleries of alpha Zr(IV) Phosphate layered inorganic nanomaterial
- determine what concentration and type of cationized Bovine Serum Albumin (BSA) and/or divalent metal ion maximizes GO Intercalation
- demonstrate if reversible GO binding occurs in an environment that simulates the human intestine

Significance:

- Noninvasive therapy
- What chemical and biological environment is necessary and sufficient for noninvasive therapy?
- (Rationale: benefits of noninvasive over invasive therapy include low toxicity)

Approach:

- Measure GO binding inside ZrP nanolayers at varied cationized BSA and divalent metal ion concentrations
- measure GO activity before and after release in an environment that simulates the intestine

Desired result:

- A specific cationized BSA or divalent metal ion maximizes GO binding
- GO activity increases after addition to a simulated intestinal environment
- Table 1 Paper summary

3.2 Approach and Rationale

Oral delivery of insulin to manage blood glucose levels in diabetic patients has been met with limited success.¹ As an alternative modality, this experiment will use the hypothesis that oral delivery of ultra-stable GO would be useful to lower glucose levels in the gastro-intestinal (GI) tract, as well as the serum glucose levels. This novel modality could aid the management of diabetes via a less painful, inexpensive, effective therapeutic method.

This new approach is non-invasive, does not require expensive hormones which need to be refrigerated which adds additional cost to the health care system, does not require repeated painful injections of insulin, or cause serious side effects such as insulin-induced diabetic coma.

The oral delivery of GO would be non-invasive, the ultra-stable preparations of GO would not require refrigeration, and these can be delivered as a safe drug in the form of a pill or capsule. When taken with a meal at proper dosage, it is unlikely to cause sudden drop in blood glucose levels and prevent serious health conditions such as diabetic coma. However the noninvasive delivery of GO as an enzyme drug would require that GO be made stable over a wide pH range (pH 1-8) and resist enzymatic degradation in the stomach and the intestines.

If someone tried to swallow a pill of glucose oxidase as this sentence is being read, it would denature in the stomach. After all, the pH of the stomach can get as low as a pH of 1 or 2.² In this experiment the goal is to get glucose oxidase to the intestines without being denatured. ZrP, the purple plates in figure one, will be used as a carrier to protect glucose oxidase, the blue circle, from denaturing in the stomach and allow it to pass to the intestines where it can be activated. Please note that ZrP is ultra-stable to even strongly acidic conditions of pH less than one and up to eight.

The Kumar group has previously shown that proteins and enzymes that are intercalated in α -ZrP are not only ultra-stable to room temperature, but also that they retained their biological activities to a significant extent.³ A similar strategy of GO intercalated in α -ZrP is expected to be highly stable to enzymatic digestion in the GI tract, but GO is a negatively charged⁴ enzyme, and its binding to the negatively charged α -ZrP⁵ is poor.³ This experiment will attempt to overcome this limitation of GO by applying a innovative strategy.

Using the principle of opposites attract, positively charged metal ions or cationized BSA will be used to decrease the unfavorable negative charge on GO and increase its binding affinity to α -ZrP. The cationized BSA or metals will serve as a glue (Fig. 1). Once glucose oxidase is intercalated, as seen on the left of the arrow, it will be protected against degradation by enzymes present in the stomach. This protection will be mostly due to the narrow galleries of the solid, which are the same size as GO's the diameter. At the acidic pH of the stomach, the cationized BSA or metal salt that is co-intercalated with GO into the galleries of α -ZrP will provide buffer

capacity and protect the protein against acid-catalyzed hydrolysis of the enzyme. For example, the metal bound COOH side chains of the aspartate and glutamate amino acid chains of GO will function as a weak acid-strong base buffer and keep the pH of the local medium surrounding the enzyme at a constant level. This constant pH level will be kept even when exposed to a highly acidic environment. The phosphate groups of the inorganic solid will also aid in buffering against the low pH of the stomach. When the pH is raised, as in the intestinal fluids (pH7-8), the enzyme is expected to be stable. Again, the enzyme is expected to convert glucose to gluconic acid and lower the glucose levels in serum and the intestines.

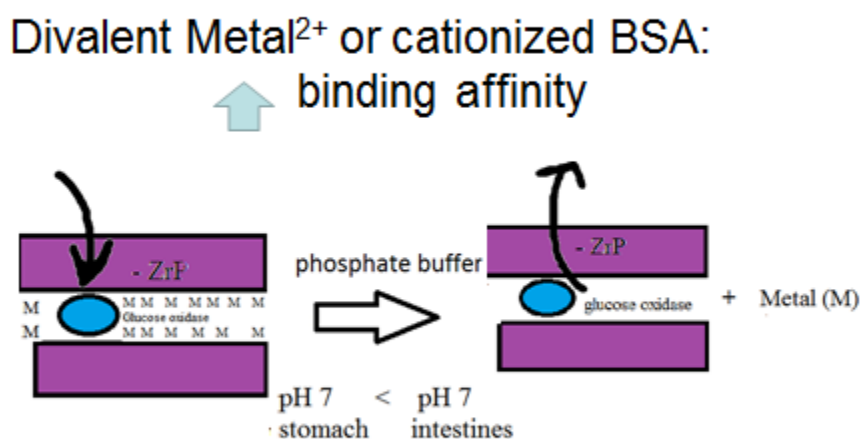


Figure 1

3.3 Applications: Chronic Disease Medication Delivery System

As described above, GO intercalation into ZrP nanolayers with a divalent metal ion or cationized BSA can serve as an alternative way to decrease serum glucose levels in diabetics. If GO is successfully intercalated into ZrP's nanolayers, it can pass the acidic stomach environment. Furthermore, if GO is then successfully released in the intestinal environment, it can convert glucose to gluconic acid and lower glucose levels in the gut. Resultantly, lower gut glucose concentrations would cause serum glucose to follow its concentration gradient and enter the gut. Thus, GO release in the intestines can potentially be used alone or in conjunction with a diabetic's insulin regimen. GO as an oral non-invasive insulin alternative to lowering serum glucose levels is especially beneficial to type one diabetics who develop insulin resistance to their insulin medications over time. Furthermore, GO could be especially beneficial to type two diabetics who are no longer responsive to oral medications and face the dilemma of having to resort to insulin injections.¹²

In this paper, GO serves as the model oral protein treatment for type one and two diabetes, however, GO can be replaced by other proteins and thus treat other diseases beyond diabetes. In addition to applications in lowering blood glucose levels in type one and two diabetics, the same overall scheme described in figure one can be applied to problems such as lowering elevated cholesterol through non-invasive oral enzyme therapy. As shown in table 2, instead of glucose oxidase being high in a patient suffering from hypercholesterolemia, the substance that would be high is cholesterol. Also, instead of using glucose oxidase, cholesterol esterase can break down cholesterol into its components and potentially result in lowering serum cholesterol levels.

Moreover, another example would be lowering the serum levels of urea in patients with kidney disease. In this case, urease can be used to degrade urea into its components carbon dioxide and ammonia. Oral delivery of urease can be beneficial for these patients and it could potentially decrease the frequency of dialysis trips to the hospital.

Problem	Elevated x at bloodstream	+ Enzyme →	Convert to y + excrete
1 Diabetes	Glucose $C_6H_{12}O_6$	Glucose oxidase	Gluconic acid
2 Hypercholesterolemia	Cholesterol	cholesterol esterase	fatty acids + cholesterol esters
3 kidney disease	urea	urease	$CO_2 + NH_3$



Table 2: Applications

3.4 Key Experimental Components:

The key to both the ZrP-GO-ionized BSA and ZrP-GO-divalent metal ion systems is demonstrating reversible binding. Binding must be reversible because the GO must first be bound inside the ZrP nanolayers while in the acidic stomach environment before it can be released in the less acidic intestine region of the GI tract. Reversible binding is tested in this experiment by measuring GO activity in environments that simulate in vivo pre intestinal, intestinal conditions. In vitro conditions are varied modeling after in vivo human sodium chloride and pH conditions.

A further key component to the ZrP-GO-divalent metal ion system in the above scheme (fig 1) is choosing the right metal ion for the co-intercalation. The optimal metal ion must become a good buffer when complexed with GO surface amino acid residues' carboxyl groups, in the low pH region. The optimal metal must also be redox inactive and non-toxic. Additionally, the optimal metal must not alter GO's catalytic activity. For example, Ca^{2+} , Mg^{2+} , and Ba^{2+} can be considered as effective and safe metal ions for this application. Once the ZrP-Metal carrier reaches the intestines, which have a pH greater than seven, the glucose oxidase should have low affinity for the metal ion. The optimal result would be that glucose oxidase will still retain its biological activity for the conversion of glucose to gluconic acid in the intestines.

4. Materials and Methods

Beta-mercaptoethanol was obtained from Fisher Scientific. Furthermore, peroxidase type 1 from horseradish (HRP) and enzyme glucose oxidase (GO from *Aspergillus niger*) were ordered and used as received from Calzyme Laboratories Incorporated. (based in California) All other materials including α -Zr(IV) phosphate (α -ZrP), metal chlorides (MgCl_2 , CaCl_2 , BaCl_2), agarose, activity plates, guaiacol, glucose, Bovine Serum Albumin (BSA), Tetraethylenepentamine (TEPA), sodium phosphate (monobasic), sodium chloride, and 1-Ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC) were provided courtesy of the University of Connecticut's Kumar Lab.

4.1 α -Zrp, GO, Cationized BSA, and Buffer Synthesis

60 μM exfoliated α -Zrp was prepared in 10 mM phosphate buffer at pH 7.004. Next, the 60 μM α -Zrp was exfoliated with TBA according to the reported method.¹³ 30 μM GO protein was also synthesized in phosphate buffer at pH 7.004 using the extinction coefficient ϵ $1.336 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.¹¹ Furthermore, ionized versions of BSA (BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)) were prepared using TEPA according to the previously accomplished method⁷ along with the extinction coefficient $43,824 \text{ M}^{-1} \text{ cm}^{-1}$.¹⁵

Buffer simulating the in vivo human intestinal environment was made. This comprised of 68.62 mM sodium chloride in pH 6.5 10 mM sodium phosphate buffer. Additionally, a control buffer of 10 mM sodium phosphate buffer with no sodium chloride and a pH of 7 was also made.

4.2 Binding Studies

Binding studies were performed according to the previously utilized method.^{7 11 16} Varied concentrations (0, 25, 50, 75, and 100 μM) of divalent metal ion (Mg (II), Ca (II), and Ba (II)) were combined with 3 μM exfoliated α -ZrP, 30 μM GO, and 10 mM sodium phosphate buffer pH 7.004. Next, samples were allowed to equilibrate for one hour at room temperature and centrifuged using a Fisher Scientific microcentrifuge set to 10000 rpm for 7 minutes. Next, UV absorbances values of supernatant samples were measured at 270 nanometers. Then, percent free GO and percent bound GO were calculated using the Scatchard equation.¹⁷ Where k_b is the binding constant, c_f is the free enzyme concentration, r is the ratio of the bound GO

concentration to the alpha ZrP concentration, and n is the number of binding sites, and K_b is the binding constant.

$$r/cf = kb(n - r) \quad (1)$$

This equation was thus used to examine GO binding to alpha ZrP. Lastly, binding figures were constructed. (see results section)

The above was repeated using varied concentrations (1, 10, 20, 30, and 40 μ M) of GO were combined with 3 μ M exfoliated alpha-ZrP, 10 mM sodium phosphate buffer pH 7.004, and 10 μ M cationized BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)).

These binding studies were done in order to find what divalent metal ion metal concentration maximizes ZrP GO intercalation and thus should be used in the release studies that followed. Furthermore, Binding studies were also done in order to find what GO concentration maximizes GO intercalation in the presence of BSA cationized to differing extents (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)). This GO concentration was thus used in the release studies that followed.

4.3 Gel Electrophoresis:

Gel electrophoresis was done to confirm the extent of cationization of BSA samples after modification with TEPA. 25 mL 40 mM Tris acetate pH 7.02 was added to 125 mg agarose, heated until the agarose was completely dissolved, and poured in a gel holder. A comb was inserted in the center of the gel and the gel was left to solidify for approximately 30 minutes. In the meantime, running buffer was made by adding 16.6 mL 40 mM Tris acetate pH 7.02 to 150 mL distilled water. Next, 10 μ L of equal concentrations (10 μ M) of all BSA samples (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)) were added to 10 μ L running buffer.

Next, the gel ran for 30 minutes at 100 Volts to allow the proteins to separate based on charge. Then, the gel was stained overnight in 20% v/v acetic acid, 0.02% m/m Coomassie Blue⁷. The following day destain (20% acetic acid⁷), isopropanol, and kimwipes were added to the gel container. After an additional 24 hours the gel was placed in a ziploc bag, imaged, and the protein bands analyzed as seen in the results section.

4.4 Zeta Potential Studies

Samples of ZrP-GO-cationized BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)) were prepared for zeta potential studies using the same concentration as listed in the binding studies above and release studies below. However, samples were not centrifuged. After sample preparation, samples were equilibrated at room temperature for approximately 45 minutes. The Brookhaven Zeta Plus zeta potential analyzer (model K01279) was used to monitor surface charge changes on exfoliated alpha ZrP in the samples of ZrP-GO-cationized BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-

TEPA (+5). Exfoliated alpha ZrP was assumed to have a surface charge and its surface charge change monitored after cationized BSA addition. 1.5 mL of each sample suspension was loaded in a 4 mL Fisher polystyrene cuvette and placed in the Brookhaven Zeta Plus instrument for analysis. Zeta potential values were calculated using the software's Smoluchowski fit and the "electrophoretic mobility" of each sample.¹¹

4.5 GO Release Studies

Samples of ZrP-GO-cationized BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)) were prepared for release studies using the same concentration as listed in the binding and zeta potential studies above. Samples were prepared in buffers that simulate differing regions of the GI tract. A control set of activities was done in 10mM sodium phosphate buffer with no sodium chloride and a pH of 7. Also, a set of activities was done on samples that simulate the in vivo human intestinal environment. This environment was simulated with 68.62 mM sodium chloride in pH 6.5 10 mM sodium phosphate buffer.¹⁸ Furthermore, activities were tested on untreated 10 μ M GO protein, GO with exfoliated alpha ZrP, and exfoliated alpha ZrP alone. The temperature of all samples was also kept at a 25 °C during activity measurements.

13 μ L of 20 μ M ZrP-GO-divalent metal ion (Mg (II), Ca (II), or Ba (II)) samples were combined with 50 μ L of 10 mM guaiacol, 4 μ L of 2 μ M HRP, and 108 μ L respective GI tract stimulatory buffers. 20 μ M GO was used for the divalent metal ion samples because this concentration was a concentration of optimal GO binding. (see binding results in binding section) Furthermore, protein was not precipitated by metal at this GO concentration. Activities were measured using the Flexstation 3 Benchtop Multi-Mode Microplate Reader. The Flexstation 3 was set to read absorbances at 2 second intervals immediately after injection with 75 μ L of .2 mM glucose. The time interval set was 120 seconds, absorbance 470 nanometers, calibration on, settle time off, and lag time set to 0 seconds. Absorbances were measured at 470 nanometers because the HRP catalyzed reaction makes a colored product with an absorption maximum at 470 nanometers.¹¹

The above activity studies were repeated after mixing 25 μ L of 10 μ M ZrP-GO-cationized BSA containing samples. (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)), 50 μ L of 10 mM guaiacol, 4 μ L of 2 μ M HRP, and 96 μ L respective GI tract stimulatory buffers. 10 μ M GO was used for the cationized BSA samples because this concentration was a concentration of optimal GO binding. (see binding results in binding section) Activity and retention graphs were then plotted using absorbance values taken over time.

5 Results and Discussion

5.1 Binding Studies

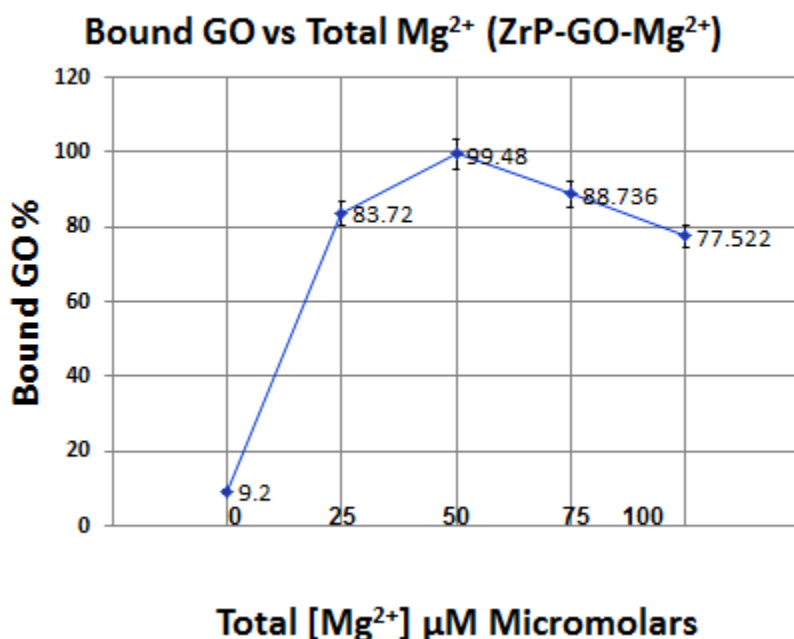


Figure 2: Binding Results of System One: Percentage GO (30 μM) binding in presence of varied concentrations (0, 25, 50, 75, and 100 μM) of divalent metal ion (Mg (II)) graphed here), 10 mM sodium phosphate buffer pH 7.0, and 3 micoM exfoliated alpha-ZrP. One divalent metal ion system (ZrP-GO- Mg^{2+}) of the three tested (ZrP-GO- Mg^{2+} , ZrP-GO- Ca^{2+} , ZrP-GO- Ba^{2+}) is displayed above. Maximum GO intercalation into alpha-ZrP galleries occurred with $[Mg^{2+}]$ 50 μM . GO intercalation increased incrementally up to $[Mg^{2+}]$ 50 μM and decreased incrementally beyond $[Mg^{2+}]$ 50 μM .

Binding studies showed all three tested divalent metal ions (Mg^{2+} , Ca^{2+} , and Ba^{2+}) increased GO intercalation into exfoliated α -Zrp galleries. Figure two displays percentage GO binding of (30 μM) of one divalent metal ion (Mg (II)) present at varied concentrations (0, 25, 50, 75, and 100 μM), 10 mM sodium phosphate buffer pH 7.0, and 3 micoM exfoliated alpha-ZrP. As seen in figure two, GO binding increases incrementally up to $[Mg^{2+}]$ 50 μM , where there is maximum GO binding. However, binding begins decreasing with each increase in metal concentration past $[Mg^{2+}]$ 50 μM . Comparing 0 μM , or when no metal was used, versus when 50 μM metal was used there is a jump from 9.2% to 99.48%. This is a substantial increase

of 11 times the GO binding due to divalent metal ion presence.

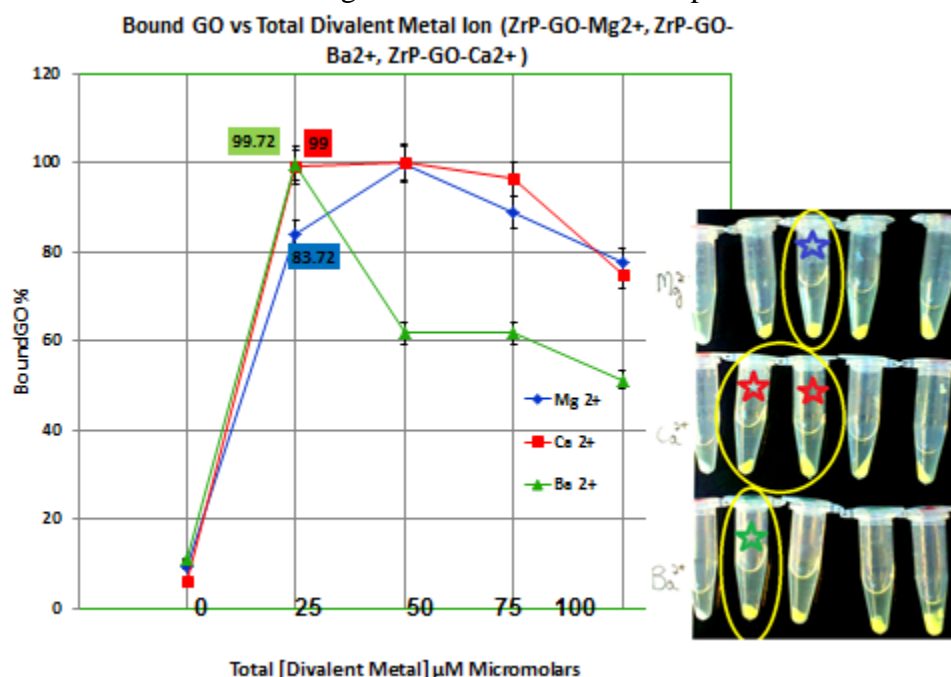


Figure 3: A side by side binding results comparison all three divalent metal ion systems is shown. (alpha-ZrP-GO-Mg²⁺, alpha-ZrP-GO-Ca²⁺, alpha-ZrP-GO-Ba²⁺) Percentage GO (30 μM) binding in presence of varied concentrations (0, 25, 50, 75, and 100 μM) of divalent metal ions (Mg²⁺, Ca²⁺, and Ba²⁺), 10 mM sodium phosphate buffer pH 7.0, and 3 micoM exfoliated alpha-ZrP. All three divalent metal ions increase intercalation of GO (or the y axis % GO binding) into the ZrP galleries. Maximum GO intercalation into exfoliated alpha ZrP galleries occurs with [Mg²⁺] 50 μM, [Ca²⁺] between 25 μM and 50 μM, and [Ba²⁺] 50 μM. Microcentrifuge tubes (left to right increasing metal concentration) correspond with and confirm data collected in the line graph.

Thus the divalent metal ion concentrations [Mg²⁺] 50 μM, [Ca²⁺] between 25 μM and 50 μM, and [Ba²⁺] 50 μM are optimal for use in a noninvasive protein drug delivery system. Future noninvasive protein drug delivery systems made of 30 μM drug of interest, 10 mM sodium phosphate buffer at pH 7.0, and 3 micoM exfoliated alpha-ZrP should use these aforementioned divalent metal ion concentrations. These concentrations should be used because they allow optimal intercalation of a drug of interest, such as GO for diabetes treatment or urease for kidney disease treatment, into alpha ZrP-GO suspensions prepared according to the above experimental conditions. (3 micoM exfoliated alpha-ZrP, 30 μM GO, and 10 mM sodium phosphate buffer pH 7.0) Optimal drug intercalation thus allows for optimal drug protection inside the galleries of alpha ZrP versus denaturing environments of the GI tract. The oral protein drug of interest can thus stay protected until it reached the segment of GI tract where release is desired.

All three metal ions are compiled on figure 3. This allowed for side by side analysis of GO binding results from all three divalent metal ion systems. (α -ZrP-GO-Mg²⁺, α -ZrP-GO-Ca²⁺, α -ZrP-GO-Ba²⁺) Figure 3 displays Barium in green, Magnesium in blue, and Calcium in yellow. Maximal percentage GO binding is boxed and displayed on the line graph along with corresponding concentration of divalent metal ion used.

All three metals increased the value of the y axis of the chart. (percentage GO binding) In other words, all three metals increased intercalation of GO into the exfoliated α -ZrP galleries. Furthermore, all three divalent metal ions have a similar behavior or follow the same pattern. There is a substantial increase in the y axis (% GO binding) at 25 and 50 μ M divalent metal ion concentrations. Moreover, the percentage GO binding quickly decreases when high metal concentrations (as represented by the right data points on the x axis) are used. Similarly, percentage GO binding again quickly decreases when low metal concentrations (as represented by the left data points on the x axis) are used.

Visual results, as seen in the microcentrifuge tubes in right of the figure, confirm graphical binding data collected and presented in the left of the figure. Each row contains a different divalent metal as labeled. The columns from left to right represent 0, 25, 50, 75, and 100 μ M of respective divalent metal ions (Mg²⁺, Ca²⁺, and Ba²⁺) with GO (30 μ M), 10 mM sodium phosphate buffer pH 7.0, and 3 micoM exfoliated α -ZrP. Furthermore, the pellet in the microcentrifuge tubes represents bound GO-ZrP and the supernatant represents unbound GO. The concentrations where maximal GO binding occurred in each α -ZrP-GO-divalent metal ion system are circled and directly matches the optimal concentrations in the line graph.

The pellet is white in the leftmost column (0 μ M each respective divalent metal ion). This means less GO (a yellow protein) is bound inside the α -ZrP-GO suspensions and thus this concentration of divalent metal ion is not optimal for a drug delivery system. Furthermore, the pellet is maximally yellow in the microcentrifuge tubes circled, meaning maximal GO (a yellow protein) is bound inside the α ZrP. However, the pellet 's yellow intensity deceases as concentrations move away from the optimal circled metal ion concentrations. This is because less GO is binding inside the ZrP galleries as concentrations move away from the optimal circled metal ion concentrations.

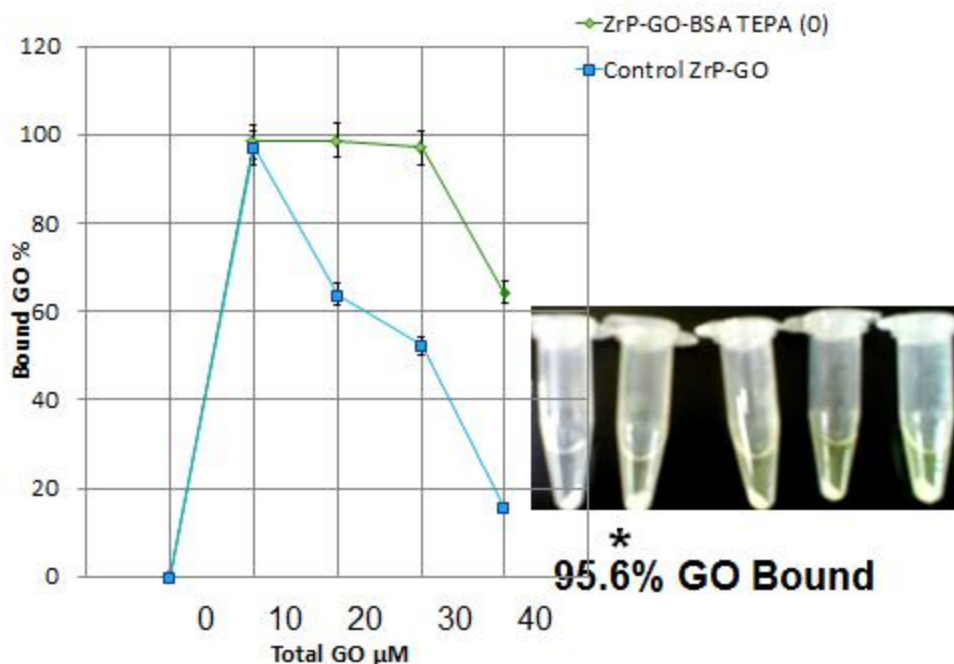


Figure 5: Binding Results of System Two: Percentage GO ($30 \mu\text{M}$) binding using varied concentrations (0, 10, 20, 30, and $40 \mu\text{M}$) of GO with 3 μM exfoliated alpha-ZrP, 10 mM sodium phosphate buffer pH 7.004, and 10 μM cationized BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)). One cationized BSA system (ZrP-GO-BSA TEPA (0)) is displayed above. 20 μM of 10 μM BSA TEPA (0) achieved maximum GO intercalation into alpha-ZrP galleries. Visual results to the right of the figure support graphical results to the left of the figure. Left to right 0, 10, 20, 30, and $40 \mu\text{M}$ of GO in presence of 3 μM exfoliated alpha-ZrP, 10 mM sodium phosphate buffer pH 7.004, and 10 μM cationized BSA. Asterisk shows 20 μM of GO in the presence of 10 μM BSA TEPA achieved 95.6% GO binding. (maximum GO intercalation)

Moving onto system two (alpha-ZrP-GO-cationized BSA), binding studies showed using a cationized form of BSA such as BSA TEPA (0) instead of native BSA increases GO intercalation into alpha ZrP galleries. Figure 5 is a graph of bound versus total GO in ZrP-GO-native BSA and in ZrP-GO-BSA TEPA (0). It compares the percentage of GO (0, 10, 20, 30, and $40 \mu\text{M}$) binding into alpha-ZrP ($3 \mu\text{M}$) galleries using 10 mM sodium phosphate buffer pH 7.004 in the presence of 10 μM native BSA versus in the presence of BSA TEPA (0). 20 μM of GO in the presence of 10 μM BSA TEPA (0) achieved maximum GO intercalation into alpha-ZrP galleries. Furthermore, GO intercalation slightly decreased as GO concentration increased beyond 20 μM .

Visual results to the right of the figure reinforce graphical results to the left of the figure. The microcentrifuge tubes from left to right contain 0, 10, 20, 30, and $40 \mu\text{M}$ of GO in the

presence of 3 μM exfoliated $\alpha\text{-ZrP}$ ($3 \mu\text{M}$), 10 mM sodium phosphate buffer pH 7.0, and 10 μM 10 μM BSA TEPA (0). The asterisk shows 20 μM of 10 μM BSA TEPA achieved maximum GO intercalation. Thus, the pellet in microcentrifuge tube above the asterisk is the most yellow because maximum GO is bound inside the $\alpha\text{-ZrP}$ galleries.

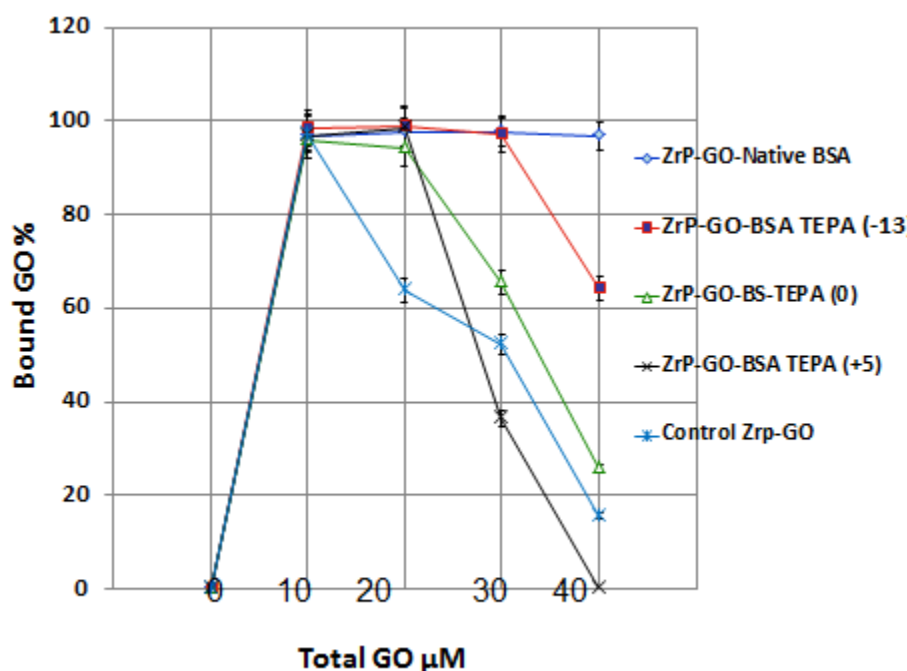


Figure 6: Percentage GO (30 μM) binding using varied concentrations (0, 10, 20, 30, and 40 μM) of GO with 3 μM exfoliated $\alpha\text{-ZrP}$, 10 mM sodium phosphate buffer pH 7.0, and 10 μM differing extents of cationized BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)). GO intercalation is maximized into ZrP with 10 and 20 μM GO no matter what form of cationized BSA was used.

Moving on, figure 6 shows a side by side comparison of GO binding with differing extents of cationized BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)). 10 μM GO in the presence of the $\alpha\text{-ZrP}$ -GO-BSA-TEPA (0) resulted in 99.97% GO binding. Thus, this green line in figure 6 corresponds to the optimal amount of charge needed on BSA.

In general, no matter what form of cationized BSA was used, increased GO Binding occurs when 10 and 20 micro molar Glucose Oxidase concentrations are used in the protein delivery system. (Zrp-GO-cationized BSA) Thus, these GO concentrations are optimal for use in a noninvasive protein drug delivery system. Future noninvasive protein drug delivery systems made of 30 μM drug of interest, 10 mM sodium phosphate buffer at pH 7.0, 3 μM exfoliated $\alpha\text{-ZrP}$, and 10 μM of cationized BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)) should use 10 and 20 micro molar drug of interest concentrations.

These concentrations should be used because they allow optimum intercalation of a drug of interest, such as GO for diabetes treatment or cholesterol esterase for hypercholesterolemia treatment, into alpha ZrP-GO suspensions prepared according to the above experimental conditions. (3 μM exfoliated alpha-ZrP, 10 cationized BSA, and 10 mM sodium phosphate buffer pH 7.0) Maximal drug intercalation thus allows for maximal drug protection inside the layers of alpha ZrP versus denaturing environments of the gastrointestinal tract. The oral enzyme drug of interest can thus stay protected until it reaches the segment of GI tract where release is desired.

5.2 Gel Electrophoresis:

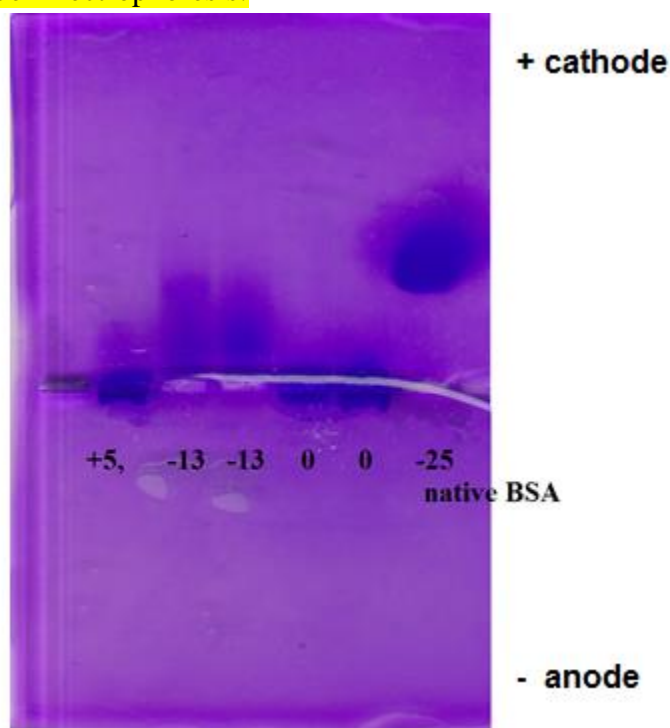


Figure 4: Cationized BSA Charge Results. Agarose gel electrophoresis confirmed successful cationization of BSA samples. Extent of charge modification was calculated by assuming native BSA has a charge of -25 and analyzing respective migration distances. The center of the diagram represents wells where protein samples were loaded. The gel was run using 40 mM tris acetate pH 7.02. Equal concentrations (10 μM) of BSA samples (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (-13), BSA-TEPA (+5)) were loaded from right to left respectively. Results right to left: native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (-13), BSA-TEPA (+5).

Gel electrophoresis confirmed the extent of cationization of BSA samples after modification with TEPA. Native BSA, assumed to have a charge of -25, moved to the positive terminal (to the top of figure 4) as expected. Furthermore, successful cationization of BSA samples was confirmed because modified BSA samples moved to negative terminal. (to the bottom of figure 4) The charges determined and listed in figure 4 were used in all other sections

of the experiment. Cationized samples of BSA were necessary because this experiment systematically tests what ionized version of BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)) intercalated into ZrP results in maximal GO binding and GO release in the intestinal region of the GI tract. These ionized versions of BSA are thus best suited for use in oral ZrP-protein drug delivery systems because they allow for optimal protection and release of drug medications for ailments ranging from diabetes to hyperchlolesterolemia.

5.3 Zeta Potential Studies:

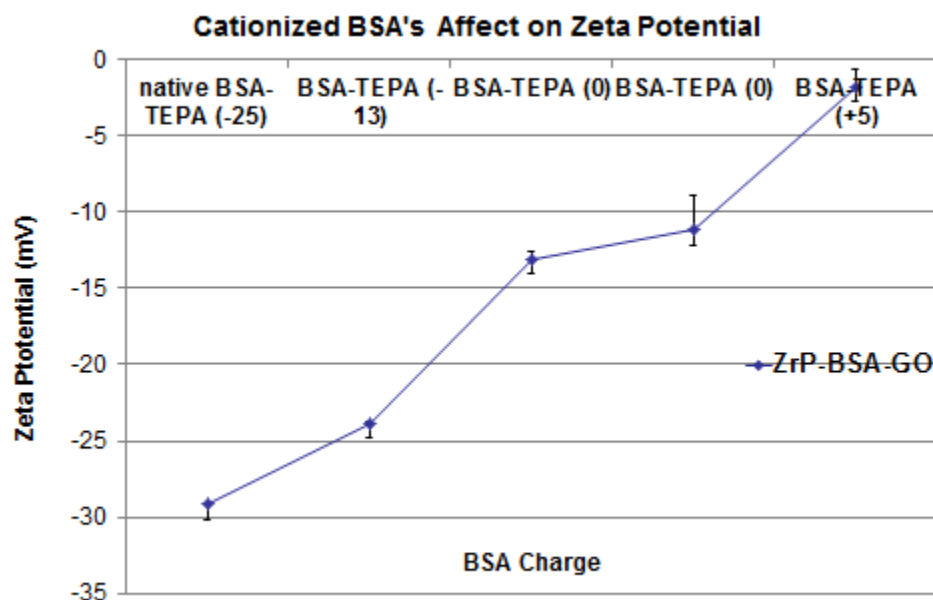


Figure 7: Zeta Potential analysis of ZrP-GO-cationized BSA (10 μ M native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)) Conditions include GO (30 μ M), 3 μ M exfoliated alpha-ZrP, 10 mM sodium phosphate buffer pH 7.004, and 10 μ M cationized BSA. ZrP's negative charge is neutralized maximally with BSA-TEPA (0) and BSA-TEPA (+5). Samples were equilibrated at room temperature for 45 minutes and not centrifuged prior to zeta potential analysis.

Samples of ZrP-GO-cationized BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)) were prepared for zeta potential studies using the same concentration as listed in the binding studies above and release studies below. Exfoliated alpha ZrP was assumed to have a surface charge and its surface charge change monitored after cationized BSA addition. In other words, native BSA serves as a control and has much more negative zeta potential value (as expected) and as compared to the cationized forms of BSA.

Zeta potential increases as BSA becomes more cationized. (figure 7 left to right) Furthermore, ZrP's negative charge is neutralized maximally with BSA-TEPA 0 and BSA-TEPA +5. Thus, zeta potential studies, as supported by agarose gel electrophoresis, confirmed successful cationization of BSA samples used in the binding and release studies of the experiment. Successful BSA cationization is critical to the experiment because this experiment

systematically tests what ionized version of BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5), intercalated into ZrP results in maximal GO binding and GO release in the GI tract's intestinal region.

This experiment hypothesizes that cationized versions of BSA in comparison to native BSA will act as a stronger glue that allows maximal GO binding. This hypothesis is based on the principle of opposite charge attraction that governs the interactions between negative GO, negative alpha-ZrP nanolayers and positive cationized BSA. It is expected that the more positively modified versions of BSA will minimize charge repulsion between negative GO and negative alpha-ZrP nanolayers. Resultantly, GO, or a similar negatively charged drug protein of interest, should better intercalate into the negative alpha-ZrP nanolayers. In summary, future drug delivery systems should intercalate negatively charged protein drug forms of interest into alpha ZrP using cationized versions of BSA instead of native versions of BSA. Optimal drug intercalation will allow for optimal drug protection as it travels down the GI tract to the desired site of release.

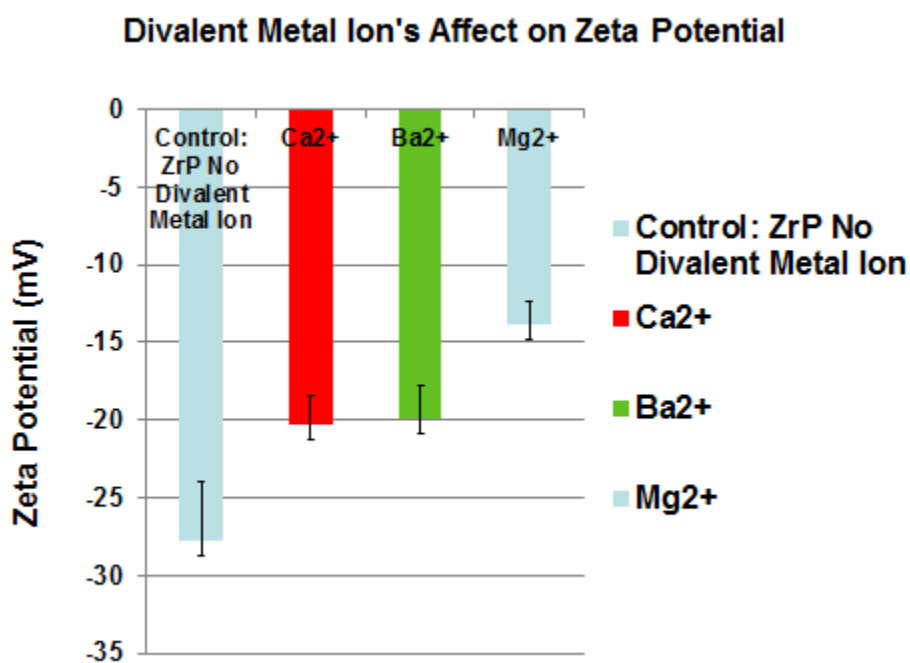


Figure 8: Zeta Potential analysis of ZrP-GO-divalent metal ion systems (Ca²⁺, Ba²⁺, Mg²⁺) Conditions include 20 μ M dialent metal ion, GO (30 μ M), 3 micoM exfoliated alpha-ZrP, 10 mM sodium phosphate buffer pH 7.004, and 10 μ M cationized BSA. The figure shows from left to right which metal ions had the most capacity to increase ZrP's charge. (left to right: least to most increase in charge: Ca²⁺, Ba²⁺, Mg²⁺) Samples were equilibrated at room temperature for 45 minutes and not centrifuged prior to zeta potential analysis.

Moving onto the divalent metal ions, figure 8 displays the zeta potential results. ZrP's negative charge is increased maximally with Mg²⁺. The figure shows from left to right which metal ions had the most capacity to increase ZrP's charge. Left to right shows from least to the most increase in charge: Ca²⁺, Ba²⁺, Mg²⁺.

Similar to the cationized versions of BSA, it is expected that alpha-ZrP present with divalent metal ions as compared to with no divalent metal ions will act as a stronger glue that allows maximal GO binding. This hypothesis is based on the principle of opposite charge attraction that governs the interactions between negative GO, negative alpha-ZrP nanolayers and positive divalent metal ions. It is expected that using positive metals will minimize charge repulsion between negative GO and negative alpha-ZrP nanolayers. Resultantly, GO, or a similar negatively charged drug protein of interest, should better intercalate into the negative alpha-ZrP nanolayers. In summary, future drug delivery systems should intercalate negatively charged protein drug forms of interest into alpha ZrP using divalent metal ions as glue. In order of least to most effective as a glue from the divalent metal ions tested is Ca^{2+} , Ba^{2+} , Mg^{2+} . Maximal medicinal drug intercalation will allow for maximal medicinal drug protection as it travels down the GI tract to the ultimate desired site of drug release.

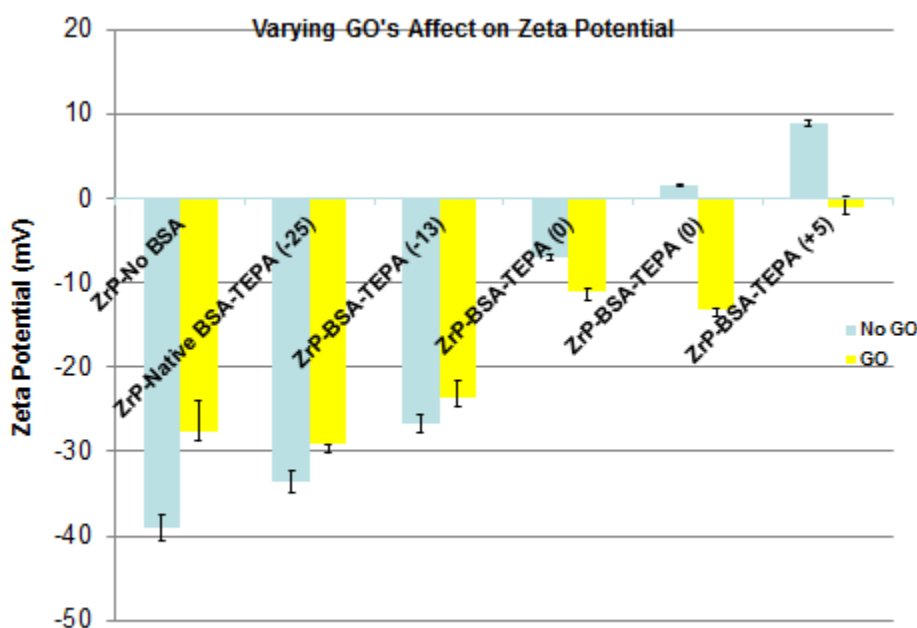


Figure 9: Zeta Potential analysis of ZrP-GO-cationized BSA (10 μM native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)) with and without GO. Conditions include GO (0 or 30 μM), 3 μM exfoliated alpha-ZrP, 10 mM sodium phosphate buffer pH 7.004, and 10 μM cationized BSA. Adding GO causes the zeta potential to be more positive with the control or no BSA, native BSA-TEPA (-25), and BSA-TEPA (-13). However, adding GO causes the zeta potential to be more negative as compared to when no GO is added with: BSA-TEPA (0) and BSA-TEPA (+5). Samples were equilibrated at room temperature for 45 minutes and not centrifuged prior to zeta potential analysis.

Results for figure 9 confirm results from figure 8. ZrP's negative charge is neutralized maximally with BSA-TEPA (0) and BSA-TEPA (+5) in figure 9. Furthermore, adding GO causes the zeta potential to be more positive with the control or no BSA, native BSA-TEPA (-

25), and BSA-TEPA (-13). Similarly, adding GO causes the zeta potential to become more positive with the divalent metal ion Ba^{2+} . (figure 10)

Zeta potential monitors the surface charge of the ZrP-GO-cationized BSA or ZrP-GO-divalent metal ion suspensions. Because the zeta potential (figure 9) of the control or no BSA, native BSA-TEPA (-25), BSA-TEPA (-13), and Ba^{2+} (figure 10) becomes more positive upon addition of GO, which is negative, GO is successfully intercalated into the ZrP galleries. If the GO had not successfully intercalated into the ZrP galleries, the zeta potential results would have become more negative upon addition of GO. This could explain why adding GO causes the zeta potential to become slightly more negative as compared to when GO is added with: BSA-TEPA (0), BSA-TEPA (+5), Mg^{2+} , and Ca^{2+} .

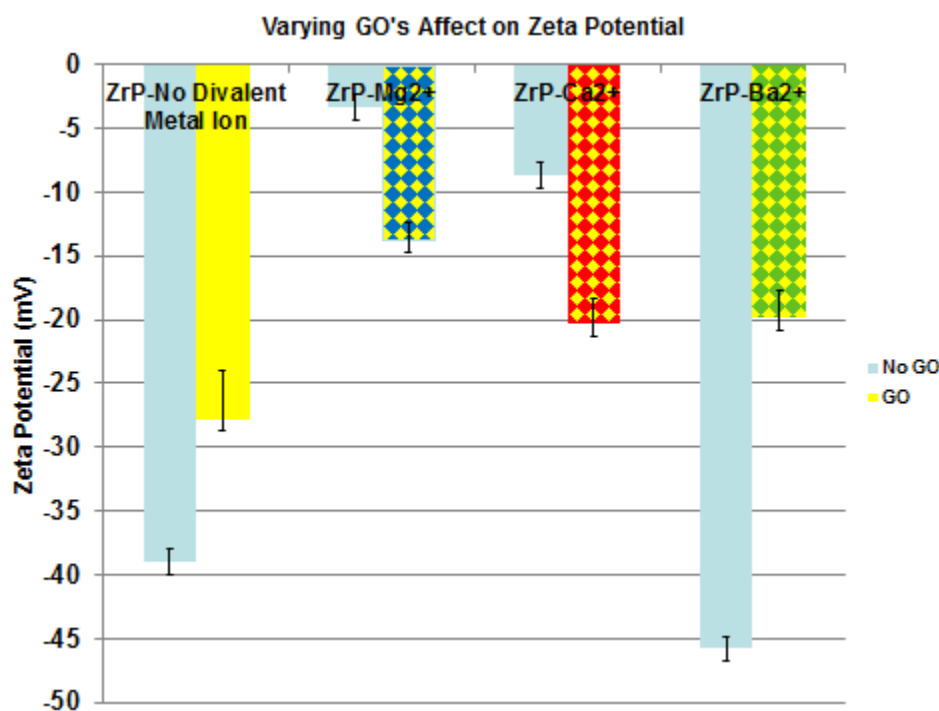


Figure 10: Zeta Potential analysis of ZrP-GO-divalent metal ions with and without GO. (30 μM GO) Conditions are 25 μM divalent metal ion (Mg (II), Ca (II), and (Ba (II)) combined with 3 μM exfoliated alpha-ZrP, and 10 mM sodium phosphate buffer pH 7.004.

Samples were equilibrated at room temperature for 45 minutes and not centrifuged prior to zeta potential analysis. Adding GO causes the zeta potential to be more positive with alpha ZrP in the presence of both the control (no BSA) and Ba^{2+} . However, adding GO causes the zeta potential to become more negative as compared to when GO is added to alpha ZrP with: Mg^{2+} and Ca^{2+} .

5.4 GO Release Studies

As stated above, the goal of the paper is to demonstrate if reversible GO binding occurs in an environment that simulates the human intestines. Reversible binding is tested by measuring GO activity before and after release in a sodium chloride, pH, and temperature controlled environment that simulates the intestine. The desired result is that a specific cationized BSA or divalent metal ion maintains or maximizes GO activity when added to a simulated intestinal GI

environment. Figures 11-14 show the activity and retention of the control. (0 g/L sodium chloride, pH 7, room temperature) Figures 15-18 show the activity and retention of the intestinal simulation (68.62 mM sodium chloride, pH 6.5, room temperature)

The percent retention represents GO's specific activity. ZrP alone has zero activity as expected because no GO is present. (figures 11-18) Activity is retained (figures 11-18) after addition of a solid. (cationized BSA or divalent metal ion)

First, the control environment results were analyzed. The control environment simulates conditions [NaCl] 0g/L, pH 7.0, 25 °C) a drug must be transported through to reach a desired release site. Retention increased upon GO addition to ZrP in presence of Mg (II), Ba (II), native BSA (-25), BSA-TEPA (-13), BSA-TEPA-(0), BSA-TEPA-(+5). (figures 12 and 14) This suggests a drug delivery system composed of drug- alpha-ZrP-Mg (II), or drug- alpha- native BSA (-25, drug- alpha-ZrP-BSA-TEPA (-13), drug- alpha-ZrP-BSA-TEPA (0), or drug- alpha-ZrP-BSA-TEPA (+5) is best to maintain a drug's activity while being transported through a similar environment (pre-Intestinal Simulation [NaCl] 0g/L, pH 7.0, 25 °C) before entering a desired site of drug release. (for example the intestines in the case of GO for diabetes treatment)

However, retention (representative of GO's specific activity) remained the same or decreased upon addition to ZrP in presence of Ca (II). This suggests a drug delivery system composed of drug- alpha-ZrP-Ca(II) is not optimal to maintain a drug's activity while being transported through a similar environment [NaCl] 0g/L, pH 7.0, °C) before entering a desired site of medicinal drug release.

Moving on, the fasted state intestinal environment simulation showed different results. Retention increased upon GO addition to ZrP in presence of Ca (II), Ba (II), native BSA (-25) and BSA-TEPA (+5). (figures 16 and 18) This suggests a drug delivery system composed of drug- alpha-ZrP-Ca (II), or drug- alpha-Ba (II), drug- alpha-ZrP-native BSA (-25), or drug- alpha-ZrP-BSA-TEPA (+5) is best to maintain a drug's activity if that drug must be released in the intestines.

However, retention (representative of GO's specific activity) remained the same or decreased upon addition to ZrP in presence of Mg (II), BSA-TEPA (0) and BSA-TEPA (-1). This suggests a drug delivery system composed of drug- alpha-ZrP-Mg(II), drug- alpha-ZrP-native BSA (-25) or drug- alpha-ZrP-BSA-TEPA (+5) is not optimal in a drug delivery system whose release site is the intestines.

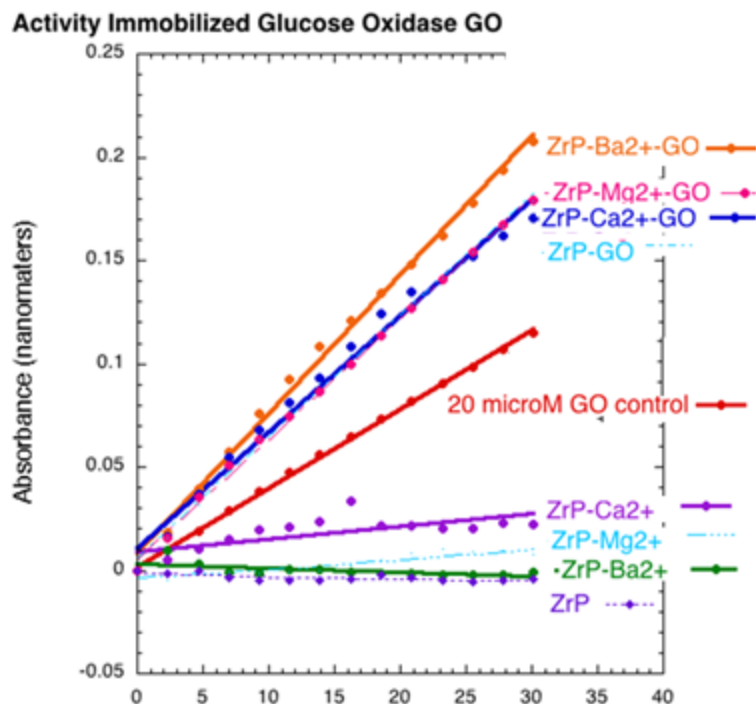


Figure 11: Pre-Intestinal Simulation ([NaCl] 0g/L, pH 7.0, room temperature): Immobilized Glucose Oxidase Activity in Presence of Divalent Metal Ions. The above slopes were taken to make figure 12 for GO retention analysis in the presence alpha-ZrP-divalent metal ion suspensions. Slopes are indicative of specific activity of GO samples. Conditions include 13 μL of 20 μM ZrP-GO-divalent metal ion (Mg (II), Ca (II), or Ba (II)) samples, 50 μL of 10 mM guaiacol, 4 μL of 2 μM HRP, and 108 μL GI tract stimulatory buffer, 20 μM GO. Absorbances were read immediately after injection with 75 μL of .2 mM glucose at 470 nanometers.

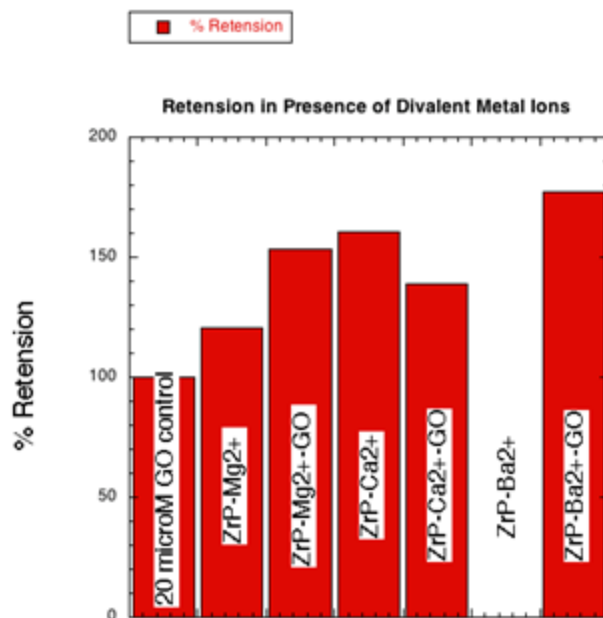


Figure 12: Pre-Intestinal Simulation ([NaCl] 0g/L, pH 7.0, room temperature): Percentage retention in the presence of divalent metal ions. Retention increased upon GO addition to ZrP in the presence of Mg (II) and Ba (II). However, retention decreased upon addition to ZrP in presence of Ca (II). Conditions include 13 μ L of 20 μ M ZrP-GO-divalent metal ion (Mg (II), Ca (II), or Ba (II)) samples, 50 μ L of 10 mM guaiacol, 4 μ L of 2 μ M HRP, and 108 μ L GI tract stimulatory buffer, 20 μ M GO. Absorbances were read immediately after injection with 75 μ L of .2 mM glucose at 470 nanometers.

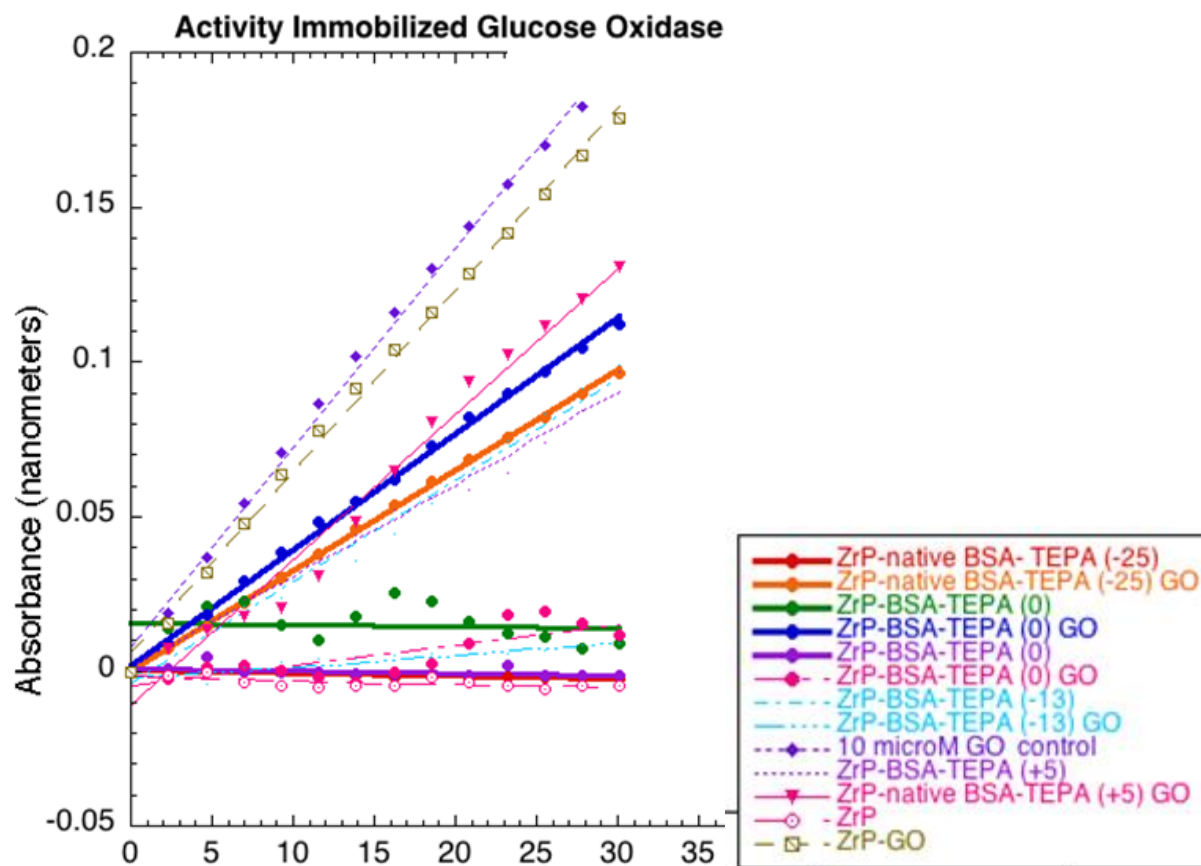


Figure 13: Pre-Intestinal Simulation (NaCl] 0g/L, pH 7.0, room temperature): Immobilized Glucose Oxidase Activity in Presence of alpha-ZrP-Cationized BSA suspensions. Slopes were taken to construct figure 14 for GO retention analysis. Slopes are indicative of specific activity of GO samples. Conditions include 25 μ L of 10 μ M ZrP-GO-cationized BSA containing samples. (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)), 50 μ L of 10 mM guaiacol, 4 μ L of 2 μ M HRP, and 96 μ L GI tract stimulatory buffer. Absorbances were read immediately after injection with 75 μ L of .2 mM glucose at 470 nanometers.

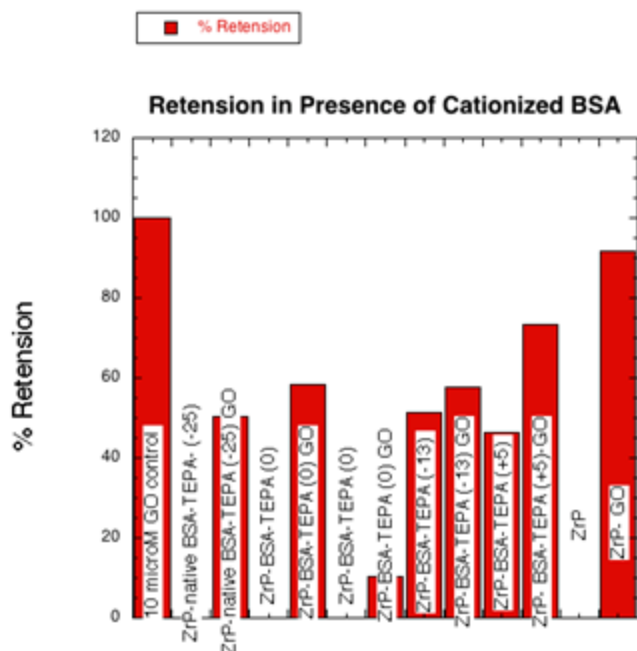


Figure 14: Pre-Intestinal Simulation ([NaCl] 0g/L, pH 7.0, room temperature) Retention in Presence of Cationized BSA. Retention increased upon GO addition to ZrP in the presence of native BSA (-25), BSA-TEPA (-13), BSA-TEPA-(0), and BSA-TEPA-(+5). Conditions include 25 μ L of 10 μ M ZrP-GO-cationized BSA containing samples. (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)), 50 μ L of 10 mM guaiacol, 4 μ L of 2 μ M HRP, and 96 μ L GI tract stimulatory buffer. Absorbances were read immediately after injection with 75 μ L of .2 mM glucose at 470 nanometers.

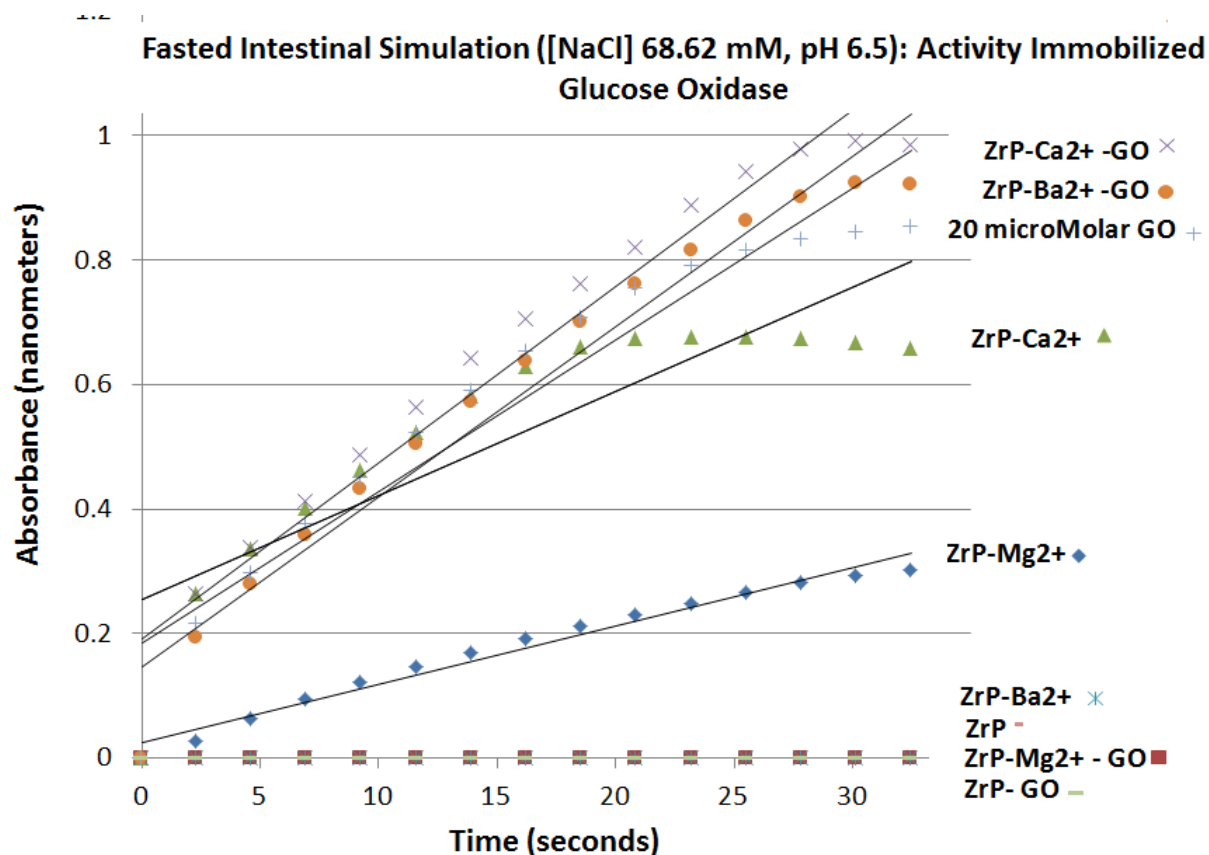


Figure 15: Fasted State Intestine Simulation ([NaCl] 8g/L, pH 7.0, room temperature): Immobilized Glucose Oxidase Activity in Presence of alpha-ZrP-divalent metal ion suspensions. Slopes were taken to construct figure 16 for GO retention analysis. Slopes indicative of specific activity of GO samples. Conditions include 13 μL of 20 μM ZrP-GO-divalent metal ion (Mg (II), Ca (II), or Ba (II)) samples, 50 μL of 10 mM guaiacol, 4 μL of 2 μM HRP, and 108 μL GI tract stimulatory buffer, 20 μM GO. Absorbances were read immediately after injection with 75 μL of .2 mM glucose at 470 nanometers.

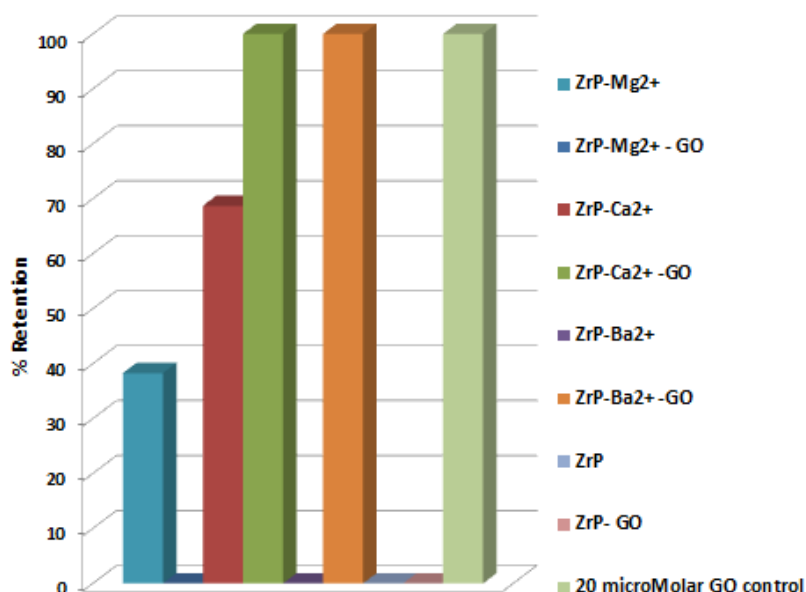


Figure 16: Fasted Intestinal Simulation ([NaCl] 68.62 mM, pH 6.5, room temperature) Percentage retention in the presence of divalent metal ions. Retention increased upon GO addition to ZrP in the presence of Ca (II) and Ba (II). However, retention decreased upon addition to ZrP in presence of Mg (II). Conditions include 13 μ L of 20 μ M ZrP-GO-divalent metal ion (Mg (II), Ca (II), or Ba (II)) samples, 50 μ L of 10 mM guaiacol, 4 μ L of 2 μ M HRP, and 108 μ L GI tract stimulatory buffer, 20 μ M GO. Absorbances were read immediately after injection with 75 μ L of .2 mM glucose at 470 nanometers.

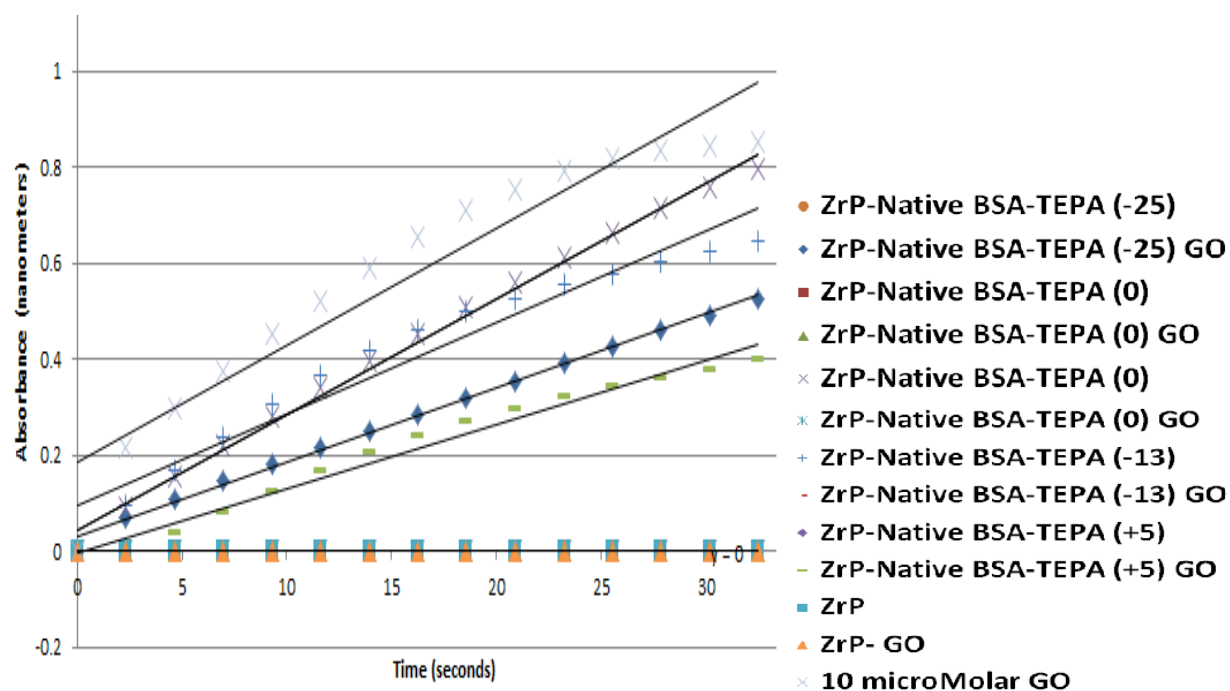


Figure 17: Fasted State Intestine Simulation ([NaCl] 8g/L, pH 7.0, room temperature): Immobilized Glucose Oxidase Activity in Presence of alpha-ZrP-Cationized BSA suspensions. The above slopes were taken to construct figure 18 for GO retention analysis. Slopes are indicative of the specific activity of GO samples. Conditions include 25 μ L of 10 μ M ZrP-GO-cationized BSA containing samples. (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)), 50 μ L of 10 mM guaiacol, 4 μ L of 2 μ M HRP, and 96 μ L GI tract stimulatory buffer. Absorbances were read immediately after injection with 75 μ L of .2 mM glucose at 470 nanometers.

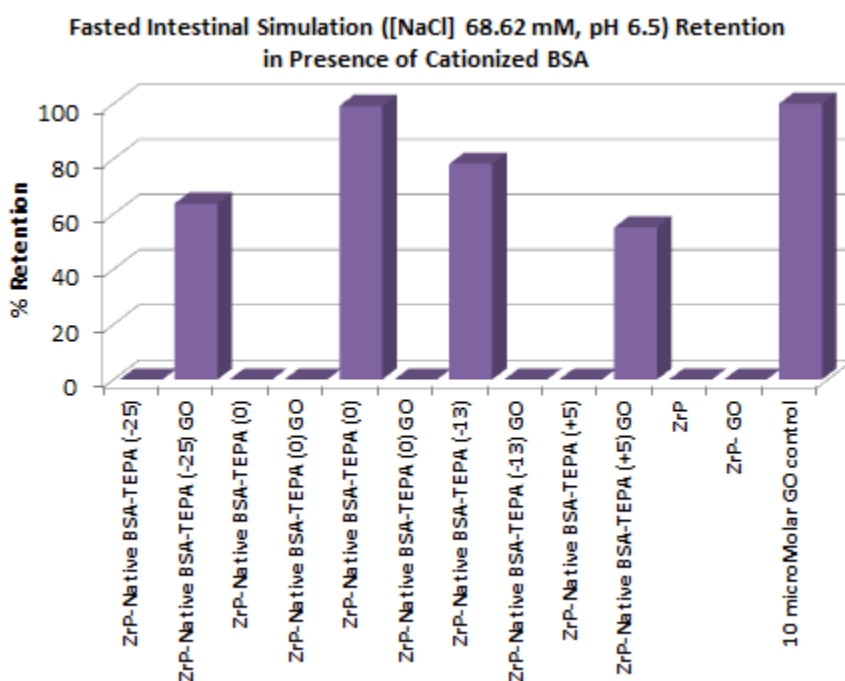


Figure 18: Fasted State Intestine Simulation ([NaCl] 8g/L, pH 7.0, room temperature): Retention in Presence of Cationized BSA. Retention increased upon GO addition to ZrP in the presence of native BSA (-25) and BSA-TEPA (+5). However, retention remained the same or decreased upon addition to ZrP in presence of BSA-TEPA (0) and BSA-TEPA (-13). Conditions include 25 μ L of 10 μ M ZrP-GO-cationized BSA containing samples. (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)), 50 μ L of 10 mM guaiacol, 4 μ L of 2 μ M HRP, and 96 μ L GI tract stimulatory buffer. Absorbances were read immediately after injection with 75 μ L of .2 mM glucose at 470 nanometers.

6 Conclusions

The above describes in detail systematic testing of what ionized version of BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5), or divalent metal ion (Mg (II), Ca (II), or Ba (II)) intercalated into ZrP results in maximal GO binding and GO release in the human intestinal environment.

Binding studies, confirmed by graphical and visual results (figure 3), showed all three tested divalent metal ions (Mg^{2+} , Ca^{2+} , and Ba^{2+}) increased GO intercalation into exfoliated α -ZrP galleries. (fig 2) Thus, the divalent metal ion concentrations [Mg^{2+}] 50 μ M, [Ca^{2+}] between 25 μ M and 50 μ M, and [Ba^{2+}] 50 μ M are optimal for use in a noninvasive protein drug delivery system. These concentrations should be used because they allow optimal intercalation of a drug of interest, such as GO for diabetes treatment or urease for kidney disease treatment, into alpha ZrP-GO suspensions prepared according to the above experimental conditions. (3 micoM exfoliated alpha-ZrP, 30 μ M GO, and 10 mM sodium phosphate buffer pH 7.0)

Furthermore, binding studies (figure 5), confirmed by graphical and visual results (figure 4), showed cationized BSA (BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)) increased GO intercalation into exfoliated α -ZrP galleries. Thus, 10 μ M cationized BSA is optimal for use in a noninvasive protein drug delivery system. This concentrations should be used because it allows for optimal intercalation of a drug of interest, such as urease for kidney disease treatment, into alpha ZrP-drug suspensions prepared according to the conditions mentioned in the results section. (3 micoM exfoliated alpha-ZrP, 10 cationized BSA, and 10 mM sodium phosphate buffer pH 7.0)

Zeta Potential studies (figure 7), as supported by agarose gel electrophoresis (figure 4), confirmed successful cationization of BSA samples utilized in the experiment. Cationized samples of BSA were necessary because this experiment systematically tests what ionized version of BSA (native BSA-TEPA (-25), BSA-TEPA (0), BSA-TEPA (-13), BSA-TEPA (+5)) intercalated into ZrP results in maximal GO binding and GO release in the intestinal region of the GI tract. These ionized versions of BSA are thus best suited for use in oral ZrP-protein drug delivery systems because they allow for optimal protection and release of drug medications for ailments ranging from hypercholerestemia to elevated serum urea levels.

Additionally, zeta potential studies showed Ca^{2+} , Ba^{2+} , and Mg^{2+} increased zeta potential or ZrP's surface charge. (ordered in least to most capacity to increase zeta potential) Similar to the cationized versions of BSA, it is thus expected that alpha-ZrP present with divalent metal ions as compared to with no divalent metal ions will act as a stronger glue that allows maximal GO binding. As stated above, the basis of this hypothesis comes from the principle of opposite charge attraction that governs the interactions between negative GO, negative alpha-ZrP nanolayers and positive divalent metal ions. It is expected that using positive metals will minimize charge repulsion between negative GO and negative alpha-ZrP nanolayers. Resultantly, GO, or a similar negatively charged drug protein of interest, should better intercalate into the negative alpha-ZrP nanolayers.

Release studies showed retention increased upon GO addition to ZrP in presence of Mg (II), Ba (II), native BSA (-25), BSA-TEPA (-13), BSA-TEPA(0), BSA-TEPA(+5). (figures 12 and 14) This suggests a drug delivery system composed of drug- alpha-ZrP-Mg (II), or drug- alpha- native BSA (-25, drug- alpha-ZrP-BSA-TEPA (-13), drug- alpha-ZrP-BSA-TEPA (0), or

drug- α -ZrP-BSA-TEPA (+5) is best to maintain a drug's activity while being transported through a similar environment (pre-Intestinal Simulation [NaCl] 0g/L, pH 7.0, 25 °C) before entering a desired site of drug release. (for example the intestines in the case of GO for diabetes treatment)

Moreover, release studies showed retention increased upon GO addition to ZrP in presence of Ca (II), Ba (II), native BSA (-25) and BSA-TEPA (+5). (figures 16 and 18) This suggests a drug delivery system composed of drug- α -ZrP-Ca (II), or drug- α -Ba (II), drug- α -ZrP-native BSA (-25), or drug- α -ZrP-BSA-TEPA (+5) is best to maintain a drug's activity if that drug must be released in the intestines.

7 Future Directions and Further Research

The binding studies above can be repeated at additional pH values that mimic specific regions of the GI tract including the mouth, large intestine, and rectum. Additionally, the activity studies can be repeated for other simulated fasted state GI tract environments in addition to the intestine such as the colon. Thus, activity results in an environment simulative of the fasted state colon (8g/L sodium chloride in 10mM pH 7.0 sodium phosphate buffer, 25°C)¹⁸ can be directly compared with the results in this paper on the fasted state intestine. Furthermore, fasted state colon GO activity results can apply to noninvasive drug delivery systems with the colon as a desired drug release site. Finally, the above studies can be repeated with another protein besides GO, the model protein in this paper. If the results of another protein share similar results to those compiled in this paper, a noninvasive drug delivery system is feasible. More importantly, an alternative to painful insulin injections, current hypercholesterolemia medications, and current kidney disease medications, may be closer than it seems.

8 References

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